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Glutathione *S*-transferase P1 (*GSTP1*) directly influences platinum drug chemosensitivity in ovarian tumour cell lines

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Background: Chemotherapy response in ovarian cancer patients is frequently compromised by drug resistance, possibly due to altered drug metabolism. Platinum drugs are metabolised by glutathione *S*-transferase P1 (*GSTP1*), which is abundantly, but variably expressed in ovarian tumours. We have created novel ovarian tumour cell line models to investigate the extent to which differential *GSTP1* expression influences chemosensitivity.

Methods: Glutathione *S*-transferase P1 was stably deleted in A2780 and expression significantly reduced in cisplatin-resistant A2780DPP cells using Mission shRNA constructs, and MTT assays used to compare chemosensitivity to chemotherapy drugs used to treat ovarian cancer. Differentially expressed genes in *GSTP1* knockdown cells were identified by Illumina HT-12 expression arrays and qRT–PCR analysis, and altered pathways predicted by MetaCore (GeneGo) analysis. Cell cycle changes were assessed by FACS analysis of PI-labelled cells and invasion and migration compared in quantitative Boyden chamber-based assays.

Results: Glutathione *S*-transferase P1 knockdown selectively influenced cisplatin and carboplatin chemosensitivity (2.3- and 4.83-fold change in IC_{50}, respectively). Cell cycle progression was unaffected, but cell invasion and migration was significantly reduced. We identified several novel *GSTP1* target genes and candidate platinum chemotherapy response biomarkers.

Conclusions: Glutathione *S*-transferase P1 has an important role in cisplatin and carboplatin metabolism in ovarian cancer cells. Inter-tumour differences in *GSTP1* expression may therefore influence response to platinum-based chemotherapy in ovarian cancer patients.

Ovarian cancer often presents at an advanced stage where treatment is rarely curative (Kristensen and Trope, 1997). Chemotherapy with platinum-based drug regimens (combining cisplatin or carboplatin with paclitaxel) can be initially effective, but longer-term treatment is frequently compromised by the development of drug-resistant disease (Vaughan *et al*, 2011). Platinum drugs are thought to act by promoting the formation of intra-strand DNA crosslinks, thus inhibiting DNA translation and replication (Eastman, 1987). Although several candidate drug resistance mechanisms, including impaired DNA repair, decreased drug uptake, increased drug efflux and detoxification, have been proposed (reviewed by Galluzzi *et al*, 2012), we are still some way from understanding the molecular basis of drug resistance, and from developing biomarkers to predict the onset and monitor the development of drug-resistant disease in cancer patients.

In recent work, we used qRT–PCR analysis to quantify inter-tumour differences in the expression of multiple candidate genes associated with disease progression and chemotherapy response in ovarian tumours (Smith *et al*, 2012). One of the most abundantly expressed genes in ovarian tumours and tumour cell lines was

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glutathione *S*-transferase P1 (*GSTP1*, EC 2.5.1.18), a polymorphic phase II drug-metabolising enzyme, which conjugates the anti-oxidant tri-peptide glutathione with many toxic hydrophobic and electrophilic xenobiotics to facilitate elimination (reviewed by Hayes and Pulford, 1995). Increased *GSTP1* expression has been reported in pre-neoplastic lesions in chemically induced animal cancer models (Satoh *et al*, 1985) and in many human tumours, relative to surrounding normal tissues (Shea *et al*, 1988). Although a useful neoplasia biomarker, it is not known whether increased *GSTP1* expression directly influences carcinogenesis, or is simply a bystander effect, where expression of this highly inducible gene is increased as part of the adaptive response mounted by the neoplastic cell. In support of a direct role in carcinogenesis, however, *GSTP1* inhibits the stress-inducible c-Jun N-terminal kinase (*JNK*) *in vitro*, and *JNK* activity is reduced in *GSTP1* null mice (Adler *et al*, 1999; Yin *et al*, 2000; Elsby *et al*, 2003). Additional roles for *GSTP1* in the regulation of genes including *TRAF2* (Wu *et al*, 2006), *CDK5* (Sun *et al*, 2011) and the *FAS* death receptor (Anathy *et al*, 2012) have also been described.

Purified *GSTP1* conjugates cisplatin *in vitro* (Hagrman *et al*, 2004), and *GSTP1* expression is increased in human tumour cell lines either inherently or made resistant to chemotherapy drugs including cisplatin and various alkylating agents (Black and Wolf, 1991; McLellan and Wolf, 1999), although a direct association linking differential glutathione conjugation with platinum drug resistance in human ovarian tumours has not been convincingly demonstrated. In support of a functional association, heterologous *GSTP1* expression in *Saccharomyces cerevisiae* (Black *et al*, 1990) or of various *GSTP1* alleles in *Escherichia Coli* influenced sensitivity to platinum and additional chemotherapy drugs (Ishimoto and Ali-Osman, 2002). In contrast, similar experiments in breast cancer MCF7 cells revealed only modest effects on platinum sensitivity (Peklak-Scott *et al*, 2008), while partial *GSTP1* knockdown in the adriamycin-resistant human colorectal cancer cell line M7609 increased sensitivity not only to the selection agent, but to cisplatin, melphalan and etoposide (Ban *et al*, 1996).

We therefore created novel ovarian tumour cell line models in which *GSTP1* expression is stably deleted, to investigate whether differential *GSTP1* expression directly influenced chemosensitivity to platinum drugs, and to other drugs routinely used in the treatment of ovarian cancer patients. We have used whole-genome transcriptional profiling analysis and various quantitative phenotypic assays to identify gene expression and associated phenotypic differences in parental and *GSTP1* knockdown cell lines.

MATERIALS AND METHODS

Ovarian tumour cell lines. A2780 and cisplatin-resistant derivative A2780DPP cells were obtained from ATCC (LGC Standards, Teddington, UK), via Cancer Research UK Cell Services. A2780 cells were maintained in RPMI-1640 media supplemented with 10% FBS, and A2780DPP cells (derived *in vitro* following long-term cisplatin selection; Behrens *et al*, 1987) in RPMI-1640 media supplemented with 15% FBS and 1 μM cisplatin. Both cell lines were maintained in 37 °C incubators, supplemented with 5% CO_2.

Creation and characterisation of stable *GSTP1* knockdown cell lines. Glutathione *S*-transferase P1 expression was stably knocked-down in A2780 and expression significantly reduced in A2780DPP cells by RNA interference using Mission shRNA plasmids (Sigma-Aldrich, Gillingham, UK). Five unique *GSTP1*-specific shRNA plasmids (clones TRCN0000083773, TRCN0000083774, TRCN0000083775, TRCN0000083776 and TRCN0000083777) and a negative control plasmid (empty vector control, SHC001) were purchased as glycerol stocks and plasmid DNA extracted using plasmid DNA maxi prep kits (Qiagen, Manchester, UK) according to the manufacturer's instructions. A2780 and A2780DPP cells (2.5×10^5 cells per well in six-well plates) were transfected with each plasmid using lipofectamine (Life Technologies, Paisley, UK), and shRNA-containing cells selected with puromycin. Individual cell colonies were picked using cloning cylinders, harvested for mRNA analysis (A2780DPP cells) or expanded to 75 cm^2 tissue culture flasks and harvested for mRNA and protein analysis (A2780 cells). *GSTP1* knockdown in A2780 cells was confirmed by qRT–PCR analysis and western blotting, and by qRT–PCR analysis in A2780DPP cells. Cell growth rates were compared by plating 1×10^5 cells from each cell line in individual wells of six-well dishes (day 0). Cells were harvested daily by trypsinisation (days 2–10) and counted using a haemocytometer.

RNA extraction and qRT–PCR analysis. Cells were grown to 80% confluency in 75 cm^2 flasks, harvested by trypsinisation, counted using a haemocytometer, and 1×10^7 cells used for RNA extraction using a Qiagen RNeasy mini kit (Qiagen), following the manufacturer's protocol for mammalian cells, with additional on column DNAse digestion (RNAse free DNAse kit, Qiagen). RNA yield and integrity were initially assessed from absorbance readings at 260 and 280 nm using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK), and confirmed using an Agilent Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent Technologies, Wokingham, UK) according to the manufacturer's guidelines.

RNA was reverse transcribed into cDNA (50 ng RNA per 20 μl RT reaction) using High Capacity RNA to cDNA kits (Life Technologies) according to the manufacturer's instructions, and the expression of *GSTP1* (Taqman probe ID Hs00168310_m1) and the loading control *18S ribosomal RNA* (Hs03003631_g1) assessed by qRT–PCR analysis, as previously described (Smith *et al*, 2012), where 20 μl individual reaction mixes (per well) contained 10 μl Taqman Universal PCR Master Mix (Life Technologies), 1 μl gene-specific Taqman probe, 1 μl cDNA and 8 μl sterile water. Each reaction was performed in triplicate and run on the Standard Real-Time PCR program on a 7900 Taqman real-time PCR system (Applied Biosystems, Warrington, UK) using pre-defined thermal cycling conditions (50 °C for 2 min, 94.5 °C for 10 min, 40 cycles of (97 °C for 30 s and 59 °C for 1 min)). Similarly, the expression of *ALX1* (Hs00232518_m1), *CDH2* (Hs00983056_m1), *FOXC1* (Hs00559473_s1), *LAYN* (Hs00379511_m1), *TM4SF* (Hs00371661_m1) and *VCAN* (Hs00171642_m1) was additionally investigated by qRT–PCR analysis in A2780 and A2780/GSTP1 cells. Analysis was performed using SDS 2.3 software (Applied Biosystems); optimal experimental baselines and thresholds were chosen for each gene, and gene expression quantitated by cycle threshold (Ct) values. Relative expression values were determined by comparing the expression of each target gene with the invariant 'loading control' *18S ribosomal RNA*, as previously described (Smith *et al*, 2012). All samples were analysed in triplicate and gene expression calculated relative to 18S ribosomal RNA ± compound error $((\text{s.d. target gene})^2 + (\text{s.d. 18S ribosomal RNA})^2)^{½}$, where s.d. = standard deviation of the mean of triplicate replicates.

Whole-genome microarray mRNA analysis in A2780 and A2780/*GSTP1* cells. RNA was prepared from A2780 and A2780/*GSTP1* cells and integrity assessed as described above. Each RNA sample was converted to biotinylated amplified cRNA using an Illumina TotalPrep RNA Amplification kit (Life Technologies) according to the manufacturer's guidelines, and cRNA quality and concentration confirmed on the Agilent Bioanalyzer 2100, as described above. cRNA samples were hybridised in triplicate on Illumina Human HT-12 BeadChip Arrays (Illumina, Little Chesterford, UK) using standard protocols optimised by the Genetics Core, Wellcome Trust Clinical Research Facility, University of Edinburgh.

Bioinformatics analysis. Gene expression data were analysed using Bioconductor 2.7 (http://bioconductor.org), running on R 2.12.1. Normalised probeset expression measures were calculated using $\log_2$ transformation and quantile normalisation using the 'Lumi' package. To identify significant differences in gene expression in A2780 and A2780/GSTP1 cells, moderated Student's *t*-tests were performed using empirical Bayes statistics in the 'Limma' package, and resulting *P*-values adjusted for multiple testing using the false discovery rate (FDR) Benjamini and Hochberg method (Smyth, 2004); probe sets with adjusted *P*-value FDR $q<0.05$ were called differentially expressed. Differentially expressed probes were also subjected to Metacore Pathway analysis (Thomson Reuters, London, UK) to identify enrichment of pathways and processes, using hyper-geometric distributions to determine the most enriched gene sets (FDR $q<0.05$).

Protein expression and western blotting. Cells for protein extraction were plated in six-well dishes, cultured until confluent, growth media removed and washed with ice-cold PBS before lysis in 0.5 ml RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40) supplemented with 2% protease inhibitor cocktail (Sigma-Aldrich). Lysed cells were centrifuged (13 000 r.p.m; 10 min) to pellet cell debris, and protein concentrations of the resulting cell supernatants determined by Bradford Assay (Bio-Rad, Hemel Hempstead, UK), relative to a standard curve prepared from serial dilutions of bovine serum albumin (0–1 mg ml^{-1}), with absorbance readings at 595 nm.

Glutathione *S*-transferase P1 expression was analysed in protein extracts from each cell line by western blotting, following SDS-PAGE. Each protein sample (40 μg) was diluted in equal volumes of 5 × sample buffer (10% SDS, 250 mM Tris-HCl (pH 6.8), 1 mg ml^{-1} bromophenol blue, 0.5 M DTT, 50% glycerol), denatured and separated by SDS-PAGE using 12% Mini-PROTEAN 3 polyacrylamide gels (Bio-Rad) in Tris-glycine buffer (25 mM Tris pH 8.3, 250 mM glycine, 0.1% SDS). Following electrophoresis, proteins were transferred to nitrocellulose membranes in Tris-glycine-methanol buffer (48 mM Tris pH 8.3, 39 mM glycine, 0.037% SDS, 10% methanol) and nonspecific antibody binding blocked by incubation for 2 h in TBST (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 5% milk powder. Membranes were then incubated overnight with a rabbit polyclonal *GSTP1* primary antibody ((Henderson *et al*, 1998), diluted 1 : 1000) or a mouse monoclonal *β-actin* antibody (sc-47778, Santa Cruz Biotechnology Inc., Heidelberg, Germany, diluted 1 : 1000), washed in PBST (PBS supplemented with 0.05% Tween-20) and incubated for 1 h with a swine anti-rabbit polyclonal secondary antibody (Dako PO399, diluted 1 : 1000, *GSTP1*, Dako, Ely, UK) or goat anti-mouse polyclonal secondary antibody (Dako PO447, diluted 1 : 1000, *β*-actin). Immunoblots were developed using an ECL-chemiluminescence kit (Merck Millipore, Watford, UK) according to the manufacturer's instructions.

Analysis of cellular glutathione levels. Total cellular glutathione (GSSG + GSH) levels were compared in A2780 and A2780/GSTP1 cells using a Glutathione Assay kit (Sigma-Aldrich) according to the manufacturer's guidelines. Cells were harvested, washed in PBS, counted using a haemocytometer and re-suspended to a final concentration of 1×10^8 cells ml^{-1} in PBS. Cells were then pelleted, de-proteinised with 5% 5-sulfosalicyclic acid and glutathione levels assessed using a kinetic assay in which catalytically active glutathione reduces 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to 5-dithiobis(2-nitrobenzoic acid) (TNB). TNB production was measured spectrophotometrically at 412 nm, with kinetic reads at 1-min intervals for 5 min, and glutathione levels extrapolated from a standard curve of serial dilutions of reduced glutathione (positive values for both GSH and GSSG are obtained in the reaction).

Ovarian cell line chemosensitivity assays. MTT assays (Mosmann, 1983) were used to compare chemosensitivity of A2780 and A2780/*GSTP1* cells to the *GSTP1* model substrate ethacrynic acid and chemotherapy drugs cisplatin, carboplatin, paclitaxel, doxorubicin, topotecan and gemcitabine. Each cell line was plated in a 96-well plate (5000 cells per well) and treated in triplicate with serial dilutions of each drug. Ethacrynic acid was used at concentrations from 2.5 to 80 μM, and chemotherapy drugs at concentrations selected to mimic typical peak plasma levels in ovarian cancer patients (range 0–200% (peak plasma); cisplatin 0–25 μM, carboplatin 0–85 μM, paclitaxel 0–32 μM, doxorubicin 0–6 μM, gemcitabine 0–190 μM and topotecan 0–56 μM; Konecny *et al*, 2000). Cells were drug treated for 72 h, media removed and 100 μl of a 0.5 mg ml^{-1} MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phenol red-free DMEM) added and cells incubated at 37 °C for 3 h. The resulting formazan crystals were solubilised in DMSO, quantitated spectrophotometrically at 570 nm and the percentage of live cells remaining following each drug treatment calculated (assigning a value of 100% to vehicle-treated cells). IC_{50} values were calculated from log dose-response curves using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

Cell invasion and migration assays. Cell invasion and migration was assessed using 24-well InnoCyte Cell Invasion and Migration Assays (Merck Millipore) according to the manufacturer's guidelines. Cell invasion was assessed by plating cells in serum-free medium in invasion chambers (8 μm membranes) coated with basement membrane matrix, which prevents non-invasive cells from passing through the membrane pores. Similarly, cell migration was compared in A2780 and A2780/GSTP1 cells by plating each cell line in serum-free medium in migration chambers. In both assays, serum-containing medium was added to each well to induce cell migration, assessed by staining cells attached to the lower side of the membrane with the fluorescent cell permeable dye Calcein-AM, and measuring fluorescence at 485 nm (excitation) and 520 nm (emission). Negative control A2780 cells were additionally treated with the anti-migratory drug Latrunculin A.

FACS analysis. Cell cycle parameters were compared in A2780 and A2780/GSTP1 cells by flow cytometry following propidium iodide labelling of cell line DNA. Cells were untreated, or treated with 12.66 μM cisplatin (to represent typical 100% patient peak plasma levels) for 1, 4 or 24 h then fixed in ice-cold 70% ethanol overnight at −20 °C, re-suspended in PBS and stained by incubation with propidium iodide (40 μg ml^{-1}, Sigma-Aldrich) and RNAse A (100 μg ml^{-1}, Sigma-Aldrich) for 30 min at 37 °C in the dark. Cell cycle parameters (10 000 cells per sample) were analysed using a FACScan flow cytometer (Becton Dickinson, Oxford, UK) and Cellquest Pro software to determine cell cycle phases and cells in sub-G_o/G_1.

Statistical analysis. All statistical tests were performed using the SPSS statistics package version 20.0 (IBM, New York, NY, USA). Independent sample *t*-tests were used to assess differences in gene expression identified by qRT–PCR analysis and invasion and migration changes in A2780 and A2780/GSTP1 cells.

RESULTS

We previously showed that *GSTP1* is abundantly expressed in human ovarian tumours, and highlighted marked inter-tumour differences in *GSTP1* expression (Smith *et al*, 2012). To investigate whether individuality in *GSTP1* expression influences chemotherapy response, we used shRNA-mediated gene silencing to stably knockdown *GSTP1* in the ovarian tumour cell line A2780, created from a chemotherapy-naive ovarian cancer patient (Behrens *et al*,

1987). A2780 cells were transfected with each of five *GSTP1*-specific Mission shRNA pLKO.1-puro plasmids (773, 774, 775, 776 and 777) and a negative control plasmid as described in Materials and Methods section. Following lipofectamine selection, multiple antibiotic-resistant colonies were screened for *GSTP1* knockdown by qRT–PCR analysis, and gene knockdown confirmed in cells transfected with plasmids 773, 775 and 776 (Figure 1A), with maximum reduction in shRNA construct 775 and 776-transfected cells. *GSTP1* protein expression was evaluated by western blotting, with complete loss of *GSTP1* expression confirmed in shRNA construct 775-transfected cells and >95% reduction in protein expression in construct 776-transfected cells (Figure 1B). GSTP1-775 knockdown cells (A2780/GSTP1) were therefore selected for use in additional experiments, following additional confirmation of increased total cellular glutathione levels (A2780 $83.16 \pm 1.67\ \mu$M and A2780/GSTP1 $116.85 \pm 4.67\ \mu$M), consistent with reduced *GSTP1* catalytic activity.

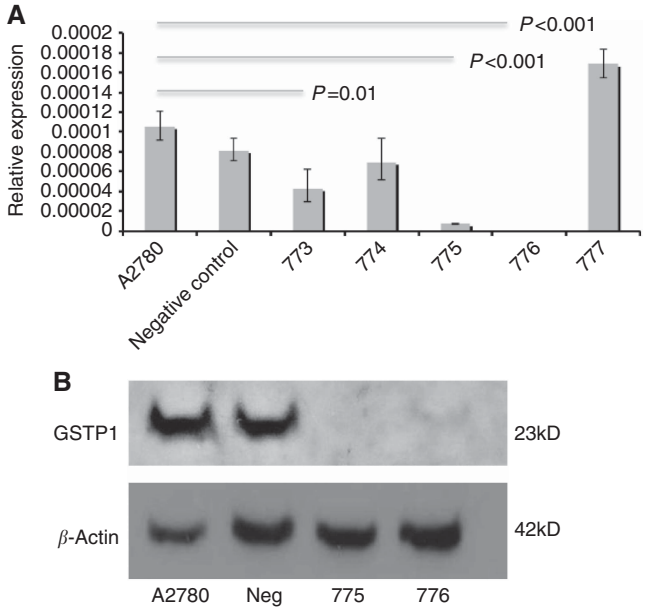

Figure 1. Generation of stable *GSTP1* knockdown cell lines. A2780 ovarian tumour cells were transfected with various *GSTP1* Mission shRNA constructs (clones 773, 774, 775, 776 and 777) and an empty vector negative control plasmid as described in Materials and Methods section. Following puromycin selection, *GSTP1* expression was compared in A2780 cells and in each novel daughter cell line (**A**) by qRT–PCR analysis, relative to *18S ribosomal RNA* (significant differences in gene expression ($P<0.05$) are highlighted) and (**B**) by western blot analysis of clones 775 and 776 to confirm loss of *GSTP1* protein expression.

MTT assays were then used to compare chemosensitivity of A2780 and A2780/GSTP1 cells to the diuretic and well-characterised *GSTP1* substrate ethacrynic acid. *GSTP1* conjugates and detoxifies ethacrynic acid (Ahokas *et al*, 1984), which was significantly more toxic to *GSTP1* null cells (1.3-fold increase in IC_{50}, $P=0.004$, Figure 2A). Sensitivity to platinum-based chemotherapy drugs was then compared in A2780 and A2780/GSTP1 cells and significant differences in chemosensitivity observed following treatment with cisplatin (2.28-fold decrease in IC_{50}, $P=0.03$, Figure 2B) and carboplatin (4.83-fold decrease in IC_{50}, $P=0.007$, Figure 2C), and a less pronounced decrease in sensitivity to paclitaxel (1.58-fold increase in IC_{50}, $P=0.02$, Figure 2D). Chemosensitivity to additional drugs used to treat ovarian cancer was also compared but no significant differences in IC_{50} values identified (doxorubicin 0.13 *vs* 0.11 μM, gemcitabine 3.32 *vs* 3.34 μM and topotecan 1.00 *vs* 1.12 μM in A2780 and A2780/GSTP1 cells, respectively (all *P*-values >0.05)). To confirm our findings, MTT assays were repeated in GSTP1-776 cells and very similar changes in cisplatin, carboplatin and paclitaxel chemosensitivity identified (data not shown).

As *GSTP1* knockdown significantly increased cisplatin and carboplatin chemosensitivity in A2780 cells, we further investigated whether *GSTP1* knockdown in the related platinum-resistant A2780DPP cell line (Behrens *et al*, 1987) could re-sensitise these drug-resistant cells to platinum-based chemotherapy. A2780DPP cells were therefore transfected with shRNA constructs 775 and 776, optimised for *GSTP1* knockdown in the experiments described

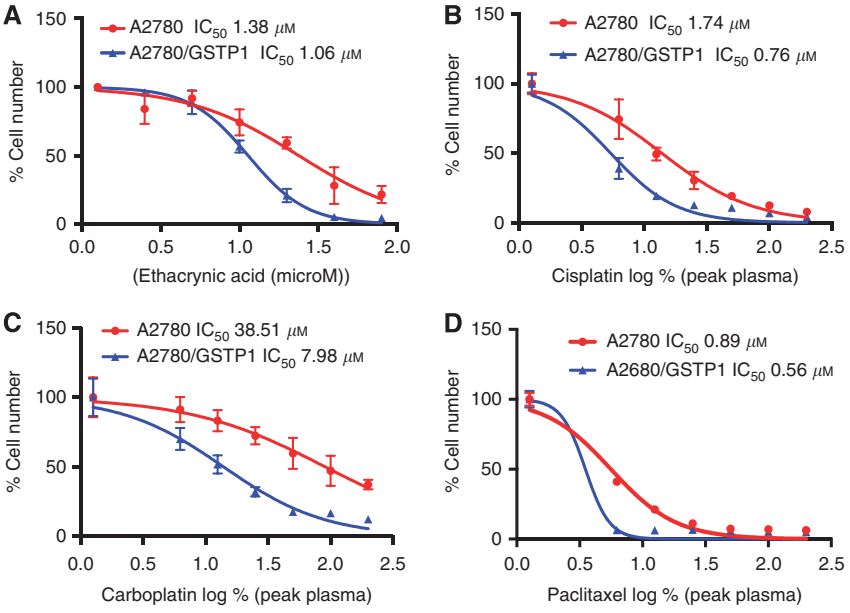

Figure 2. Chemosensitivity changes induced by stable *GSTP1* knockdown in A2780 cells. MTT cytotoxicity assays were used to compare chemosensitivity to (**A**) ethacrynic acid, (**B**) cisplatin, (**C**) carboplatin and (**D**) paclitaxel in A2780 and A2780/GSTP1 cells as described in Materials and Methods section. Cisplatin, carboplatin and paclitaxel were used at doses representative of typical peak plasma levels in ovarian cancer patients. Each assay was performed in triplicate—mean data from three independent experimental replicates is illustrated.

above. In contrast to drug-sensitive A2780 cells, however, although puromycin-resistant colonies were initially formed in A2780DPP cells in multiple replicate experiments, the colonies formed were relatively small (10–20 cells per colony) and were viable for only 48 h (Figure 3A). As our control transfections resulted in viable colony formation in both A2780 and A2780DPP cells, we hypothesised that *GSTP1* knockdown may be lethal in A2780DPP cells, which are grown in medium containing 1 μM cisplatin to maintain the drug-resistant phenotype. To confirm *GSTP1* knockdown in A2780DPP cells, we isolated individual puromycin-resistant colonies 24 h after colony formation using cloning cylinders, and extracted sufficient RNA to confirm *GSTP1* knockdown by qRT–PCR analysis (Figure 3B). Unfortunately, we were unable to harvest sufficient A2780DPP/GSTP1 knockdown cells to perform western blotting, cytotoxicity assays or more detailed phenotypic characterisation, but our confirmation of *GSTP1* knockdown, together with selective platinum toxicity in puromycin-resistant clones is consistent with an essential role for *GSTP1* in glutathione conjugation and cisplatin detoxification. We further attempted to confirm this hypothesis by knocking down *GSTP1* in A2780DPP cells grown in the absence of cisplatin selection, but found that control untransfected A2780DPP cells did not retain a stable drug-resistant phenotype over the 6-week experimental period in the absence of cisplatin selection (data not shown).

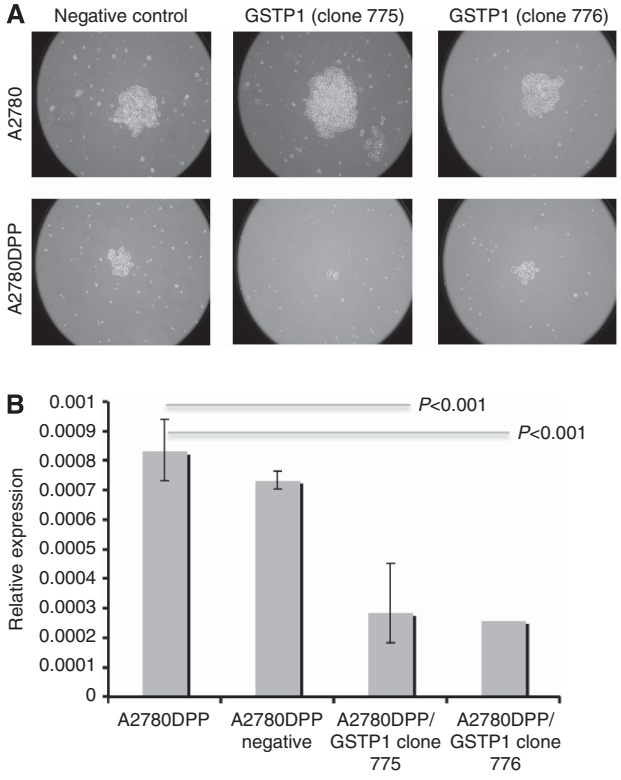

Figure 3. *GSTP1* knockdown is toxic to cisplatin-resistant A2780DPP cells. A2780 and cisplatin-resistant A2780DPP cells were transfected with *GSTP1* Mission shRNA plasmids 775 and 776 or an empty vector negative control plasmid, as described in Materials and Methods section, and *GSTP1* knockdown cells identified by puromycin selection. (**A**) Viable puromycin-resistant colonies were obtained from all transfections in A2780 cells, but only from A2780DPP cells transfected with an empty vector negative control plasmid. (**B**) qRT–PCR analysis was used to confirm *GSTP1* knockdown in A2780DPP cells (illustrated relative to the expression of *18S ribosomal RNA*) following transfection of *GSTP1* shRNA clones. Cells from small colonies with limited viability were collected using cloning cylinders, and RNA extracted as described in Materials and Methods section.

To investigate the cellular phenotypes resulting from *GSTP1* knockdown in A2780 cells, Illumina HT-12 Expression BeadChip Arrays were used to compare gene expression in A2780 and A2780/GSTP1 cells. Each HT-12 array contains >47 000 unique probe sets, corresponding to >28 000 coding transcripts; 2671 probes were significantly more highly expressed, and 2717 probes less highly expressed in A2780/GSTP1 cells. Of these, ⩾2-fold differences in gene expression were identified for 336 gene transcripts—the most significantly differentially expressed genes are summarised in Supplementary Table 1 (Supplementary Information), and changes in gene expression predicted by BeadChip Array analysis confirmed by qRT–PCR analysis for selected up- and downregulated genes (Figure 4). It was of particular interest to note that no compensatory changes in additional GST isoforms were identified in A2780/GSTP1 cells.

Pathway and process enrichment analysis was then used to identify common differentially regulated pathways or processes in A2780 and A2780/GSTP1 cells (Supplementary Table 2). Consistent with these predictions, the most significant differences in gene expression were seen for genes (e.g., N-cadherin (CDH2), versican (VCAN), L6 cell surface antigen (TM4SF1) and layilin (LAYN)) associated with cell invasion, migration, metastasis and the epithelial–mesenchymal transition. Consistent with known functions of the GSTs, several differentially regulated pathways and processes were associated with lipid metabolism and with the oxidative stress response, while additional interesting associations suggested altered cell cycle regulation and differences in apoptosis, cell invasion and migration. We therefore used quantitative cell growth, invasion and migration assays and FACS analysis to compare A2780 and A2780/GSTP1 cell phenotypes. We did not observe significant differences in cell growth rates, where A2780 and A2780/GSTP1 cells had doubling times of 0.91 and 1.03 days, respectively ($P = 0.462$), or in cell cycle parameters, assessed by FACS analysis of propidium iodide-labelled untreated cells and stressed cells acutely treated with cisplatin (Figure 5A). In contrast, consistent with the gene expression and pathway/process differences described above, we found that both cell invasion (Figure 5B) and migration (Figure 5C) was significantly reduced in A2780/GSTP1 cells.

DISCUSSION

Conjugation of glutathione with platinum-based chemotherapy drugs is an important detoxification mechanism, which promotes drug clearance, limits the formation of DNA crosslinks and reduces toxicity (Peklak-Scott *et al*, 2008). It is therefore logical that the expression of glutathione-conjugating enzymes including *GSTP1* is increased as an adaptive response in drug-resistant tumour cells (Black and Wolf, 1991; McLellan and Wolf, 1999) and that reduced *GSTP1* activity may influence chemosensitivity. Several studies, however, have described increased *GSTP1* expression in drug-resistant cell lines following exposure to drugs which are not detoxified by glutathione conjugation (Wang *et al*, 1989), or which are not *GSTP1* substrates (Tew, 1994)—a direct role for *GSTP1* in platinum chemosensitivity therefore remains to be unequivocally established.

We have shown for the first time that stable deletion of *GSTP1* in A2780 ovarian tumour cells significantly and selectively increases sensitivity to cisplatin and carboplatin, drugs routinely used to treat ovarian cancer, a notoriously drug-resistant and clinically intractable disease. Importantly, loss of *GSTP1* expression in the cisplatin-resistant A2780 subline A2780DPP was toxic to the cells in the presence of cisplatin, consistent with an important catalytic role for *GSTP1* in glutathione conjugation in the detoxification pathway of platinum drugs. Consistent with this hypothesis, we found significant differences in intracellular glutathione levels and in sensitivity to the GST substrate ethacrynic acid in *GSTP1* null cells. Although the role of *GSTP1* in ovarian

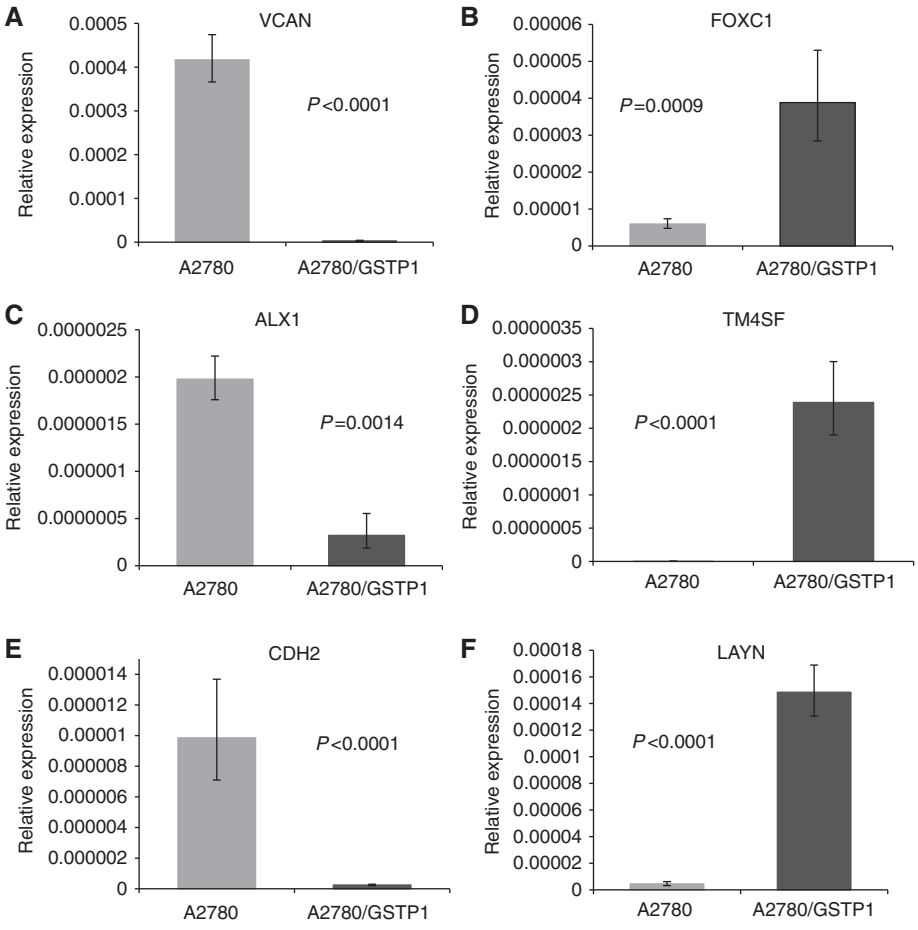

Figure 4. qRT–PCR analysis confirms gene expression changes predicted by Illumina Beadchip microarray analysis of A2780 and A2780/GSTP1 cells. qRT–PCR was performed, as described in Materials and Methods section, to confirm differential expression of selected genes predicted by comparative Illumina HT-12 Beadchip Array analysis to be differentially expressed in A2780 and A2780/GSTP1 cells—(**A**) VCAN, (**B**) FOXC1, (**C**) ALX1, (**D**) TM4SF, (**E**) CDH2 and (**F**) LAYN. All samples were analysed in triplicate; gene expression is illustrated relative to the expression of *18S ribosomal RNA*.

tumour chemosensitivity has not been studied, transient siRNA-mediated *GSTP1* knockdown in a diverse panel of leukaemia and lymphoma cell lines has recently also been reported to influence cisplatin sensitivity (Chen *et al*, 2013).

Glutathione *S*-transferase P1 has also been proposed to have a non-catalytic role in promoting cell proliferation by binding to and inhibiting *JNK* (Adler *et al*, 1999)—*JNK* activity is increased when *GSTP1* activity is reduced, either by small molecule *GSTP1* inhibitors or in *GSTP1* null mice (Henderson *et al*, 1998). It is logical to propose, therefore, that stable *GSTP1* deletion in A2780 ovarian tumour cells may result in *JNK* activation. Consistent with this hypothesis, interrogation of our gene expression microarray data sets revealed increased expression of several *JNK* regulatory genes including the toxicity and *JNK* inducer *TAJ* (*TNFRSF19*, 8.33-fold, adjusted *P*-value 1.25×10^{-18}, Supplementary Table 1), and *JNK* target genes including the p53-inducible proteins *TP53I3* (*PIG3*, 5.02-fold, adjusted *P*-value 1.73×10^{-16}) and *CDKN1A* (*p21*, 4.87-fold, adjusted *P*-value 9.62×10^{-15}) in *GSTP1* knockdown cells. Although many of the genes up- and downregulated by *GSTP1* deletion are associated with the *RAS/MAPK* pathway (Supplementary Table 1), we did not detect a significant difference in *RAS/MAPK* pathway activation, using a quantitative *RAF-1*-binding/Ras GTPase ELISA to assess pathway activation in *GSTP1* knockdown cells (data not shown). In contrast, several differentially expressed genes (e.g., *CDH2*) and associated pathways and processes suggested that *GSTP1* knockdown may significantly inhibit cell invasion and migration—these predictions were confirmed experimentally, and are consistent with the hypothesis that increased *GSTP1* expression promotes neoplastic transformation and the development of drug resistance. We were therefore surprised to find that *GSTP1* knockdown did not significantly influence cell growth rate or alter cell cycle progression in A2780 cells, although similar findings have been reported in a series of ovarian cancer cell lines made resistant to cisplatin, carboplatin and paclitaxel (Li *et al*, 2004), suggesting limitations of the use of immortalised cancer cell line models. Similarly, and consistent with a previous report of comparable *GSTP1* expression in A2780 cells and the cisplatin-resistant derivatives C70 and C200 (Townsend *et al*, 2002), we did not detect a significant increase in *GSTP1* expression in platinum-resistant A2780DPP cells. In contrast, increased *GSTP1* expression has been described in many drug-resistant cell lines (Kuroda *et al*, 1991; Kotoh *et al*, 1997; Tozawa *et al*, 2008; Yang *et al*, 2009), including ovarian tumour lines resistant to platinum drugs (Lewis *et al*, 1988; Parekh and Simpkins, 1996; Yanagie *et al*, 2009). Recent data from our own laboratory, where we see increased *GSTP1* expression in a novel drug-resistant A2780 subline immediately following *de novo* platinum selection, further support the hypothesis that increased *GSTP1* expression may not be maintained in long-term culture of immortalised tumour cells. In light of these concerns, we are currently extending our analysis to additional immortalised cell lines, with different histologies and genetic backgrounds and to primary cell lines derived from ascites from drug-sensitive and drug-resistant ovarian cancer patients.

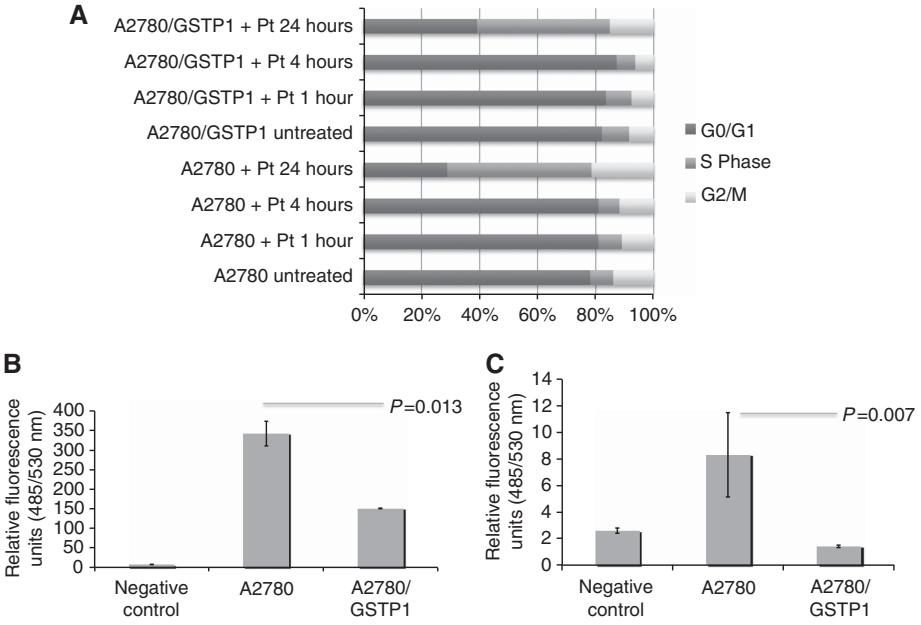

Figure 5. Phenotypic comparison of A2780 and A2780/GSTP1 knockdown cells. (**A**) FACS analysis of PI-labelled cells was used, as described in Materials and Methods section, to compare cell cycle progression of A2780 and A2780/GSTP1 cells, before and following cisplatin challenge. Each sample was analysed in triplicate—the relative proportions of cells in each phase of the cell cycle at each time point is illustrated. Boyden chamber-based (**B**) invasion (using cell culture media supplemented with 10% FBS as chemoattractant) and (**C**) migration (ECMatrix) assays were then used to compare predicted phenotypes in negative (Latrunculin A-treated) controls, A2780 and A2780/GSTP1 cells as described in Materials and Methods section.

Glutathione *S*-transferase P1 expression in cancer patients may also be influenced by the presence of allelic variants—*GSTP1* $Ile_{105}Val$ (rs1695) and *GSTP1* $Ala_{114}Val$ (rs1138272), with homozygote rare allele frequencies of approximately 12% and 2% in the Caucasian population, respectively (Zimniak *et al*, 1994; Ali-Osman *et al*, 1997; Harries *et al*, 1997; Sachse *et al*, 2002). Unlike *GSTM1* and *GSTT1*, however, where common polymorphisms result in complete gene deletions (Board, 1981), variant *GSTP1* alleles differ from the consensus reference sequence by single amino-acid substitutions, resulting in less pronounced, less frequent and less well-characterised phenotypes (Peklak-Scott *et al*, 2008). In contrast, however, our recent gene expression profiling experiments in human ovarian tumours describe marked (>70-fold) inter-tumour differences in *GSTP1* expression, suggesting that individuality in glutathione-conjugating activity, although not genetically determined, could significantly influence response to platinum-based chemotherapy in ovarian cancer patients.

Studying the influence of inter-individual differences in *GSTP1* expression on disease progression or chemotherapy response in ovarian cancer patients is challenging—the disease is frequently diagnosed at an advanced stage, limiting the availability of matched normal and tumour samples, and serial matched drug-sensitive and drug-resistant tumour biopsies are rarely available. Increased *GSTP1* expression in ovarian tumours ($n=41$), relative to unmatched healthy ovarian samples ($n=12$) and benign tumours ($n=25$) has been described in a small Chinese study (Cheng *et al*, 2000) and, consistent with our findings, *GSTP1* expression in ovarian cyst fluid has been correlated with higher relapse rates following platinum-based chemotherapy (Boss *et al*, 2001). In similar studies, *GSTP1* expression in ovarian cyst fluid correlated both with serum CA125 levels and reduced patient survival (Kolwijck *et al*, 2009), while in a small series ($n=30$) of matched first and second-look laparotomies, increased *GSTP1* expression was associated with disease progression, assessed by both more frequent relapses and reduced survival (Surowiak *et al*, 2005).

Confirmation of a direct role for *GSTP1* in chemotherapy response is important, not only in the prediction of response to platinum-based chemotherapy, but as a candidate response biomarker for new generation chemotherapy drugs, designed to exploit increased *GSTP1* expression in tumours relative to surrounding normal tissues. For example, TLK-286 (Telcyta, canfosfamide) was identified as the lead candidate in a rationally designed series of selectively toxic glutathione analogues (Lyttle *et al*, 1994), which continues to be evaluated both as a single agent and in combination chemotherapy in phase II and III clinical trials in ovarian cancer and other solid tumours. TLK-286 is metabolically activated by *GSTP1* and cytotoxicity has been correlated with *GSTP1* expression (Rosario *et al*, 2000; Dourado *et al*, 2013). Increased *GSTP1* expression in tumours and in drug-resistant cells is also the target of a new class of GST suicide inhibitors, including 7-nitro-2,1,3-benzoxadiazole (NBDHEX) (Ricci *et al*, 2005), which acts to induce apoptosis by promoting dissociation of the *GSTP1/JNK1* complex in leukaemia cell lines (Turella *et al*, 2005). Similar effects were recently observed in mesothelioma cell lines, where NBDHEX synergised with cisplatin (De Luca *et al*, 2013).

We have described marked inter-tumour differences in *GSTP1* expression but, unlike fibroblast growth factor (*FGF*) family genes, pre-treatment tumour *GSTP1* expression was not influenced by tumour histology or associated with altered survival (Smith *et al*, 2012). Our study did not have sufficient power to perform a meaningful assessment of the potential role of *GSTP1* in chemotherapy response, although we believe that lack of routine access to comparable clinical biopsy or tumour samples from ovarian cancer patients pre- and post-treatment may significantly limit tumour biomarker utility. It is interesting to note, however, that post-chemotherapy *GSTP1* expression was associated with progression-free survival in a small study ($n=41$ patients) where pre- and post-chemotherapy biopsies were available (Saip *et al*, 2005). We are therefore currently recruiting ovarian cancer patients to clinical studies in which we are collecting serial serum and ascites samples from matched drug-sensitive and drug-resistant patients for

quantitative biomarker profiling. *GSTP1* expression has previously been investigated by immunostaining in ovarian ascites samples, and shown to correlate with both primary tumour expression and cisplatin chemosensitivity (Kase *et al*, 1998). Our microarray data sets provide numerous examples of *GSTP1*-dependent gene expression changes, and may therefore allow us to identify additional biomarkers, which correlate with *GSTP1* activity. Additional candidate *GSTP1* biomarkers have recently identified in studies describing an inverse association between *GSTP1* expression, chemosensitivity and expression of the regulatory microRNA *miR-513a-3p* in cisplatin-resistant A549 lung cancer cells (Zhang *et al*, 2012). Similarly, interleukin-6 (*IL-6*) production has been shown to be elevated in both serum and ascites samples from ovarian cancer patients and to be inversely associated with platinum chemosensitivity and survival (Scambia *et al*, 1995). Of obvious relevance to our findings, *GSTP1* has recently been shown to be an *IL-6* target gene (Wang *et al*, 2010), while inhibition of *GSTP1* activity by an *IL-6* or IL-6 receptor mAb correlates with increased platinum (and paclitaxel) sensitivity in renal cancer cells (Mizutani *et al*, 1995). It will therefore be of particular interest to compare GSTP1 and IL-6 levels in serum and ascites samples from drug-sensitive and drug-resistant ovarian cancer patients.

In summary, and consistent with the findings of a recent similar study in mesothelioma (Chen *et al*, 2014), we have shown that *GSTP1* selectively influences sensitivity to cisplatin and carboplatin in ovarian tumour cells. Additional studies to evaluate the role of *GSTP1* and co-regulated genes as clinical response biomarkers of disease progression and platinum chemosensitivity in ovarian cancer patients are underway in our laboratory.

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